ZEB1 Enhances Transendothelial Migration and Represses the Epithelial Phenotype of Prostate Cancer Cells

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Metastatic colonization involves cancer cell lodgment or adherence in the microvasculature and subsequent migration of those cells across the endothelium into a secondary organ site. To study this process further, we analyzed transendothelial migration of human PC-3 prostate cancer cells in vitro. We isolated a subpopulation of cells, TEM4-18, that crossed an endothelial barrier more efficiently, but surprisingly, were less invasive than parental PC-3 cells in other contexts in vitro. Importantly, TEM4-18 cells were more aggressive than PC-3 cells in a murine metastatic colonization model. Microarray and FACS analysis of these cells showed that the expression of many genes previously associated with leukocyte trafficking and cancer cell extravasation were either unchanged or down-regulated. Instead, TEM4-18 cells exhibited characteristic molecular markers of an epithelial-to-mesenchymal transition (EMT), including frank loss of E-cadherin expression and up-regulation of the E-cadherin repressor ZEB1. Silencing ZEB1 in TEM4-18 cells resulted in increased E-cadherin and reduced transendothelial migration. TEM4-18 cells also express N-cadherin, which was found to be necessary, but not sufficient for increased transendothelial migration. Our results extend the role of EMT in metastasis to transendothelial migration and implicate ZEB1 and N-cadherin in this process in prostate cancer cells.

INTRODUCTION

Metastatic prostate cancer is a lethal disease and the second most frequent cause of cancer-related mortality in men in the United States (Jemal *et al.*, 2008). Unfortunately, for such a prevalent disease, much remains unknown about the cellular and molecular mechanisms underlying prostate cancer metastasis. Extravasation, a step within the metastatic cascade, is the process whereby cancer cells exit the circulation via migration through an endothelial monolayer into the parenchyma of a secondary organ site (Wood, 1958). For extravasation to occur, cancer cells, perhaps associated with a thrombus, must first contact the microvascular endothelium, becoming entrapped in small-diameter vessels or adhering specifically to the luminal surface of the endothelium (Warren and Vales, 1972; Kramer and Nicolson, 1979). Some evidence suggests that extravasation is an efficient process, whereas other studies indicate that in some cases it might not occur at all because cancer cells proliferate intraluminally before rupturing microvessels (Crissman *et al.*, 1985; Lapis *et al.*, 1988; Luzzi *et al.*, 1998). Adding to this complexity is that the mechanism of extravasation may depend on the tumor type and vascular bed involved. Thus, as with many other aspects of metastasis, questions remain about

the basic mechanisms and the overall role of extravasation in the metastatic cascade.

The passage of cancer cells across the endothelium, or transendothelial migration, is thought to be conceptually similar to leukocyte diapedesis (for a recent review see Miles *et al.*, 2008). The extent to which this is true remains controversial, but several classes of molecules that mediate diapedesis, including chemokines and their receptors, E-selectin and integrins, have also been implicated in cancer cell extravasation and metastasis (Giavazzi *et al.*, 1993; Muller *et al.*, 2001; Voura *et al.*, 2001). Additionally, cell junctional proteins including cadherins have been implicated in melanoma cell transendothelial migration (Qi *et al.*, 2005). Although leukocytes are thought to primarily utilize paracellular or transcellular migration, cancer cells may also induce retraction of endothelial cells or otherwise utilize mechanisms that increase vascular permeability (Lapis *et al.*, 1988; Lee *et al.*, 2003; Padua *et al.*, 2008). However, how the various molecules involved in adhesion to and activation of the endothelium, cytoskeletal rearrangements involved in the motility of both cancer and endothelial cells, and cancer cell invasion of the subendothelial matrix are orchestrated in cancer cell extravasation remains poorly understood.

The use of in vitro transwell model systems has helped to elucidate important molecular and cellular interactions that are required for transendothelial migration of cancer cells (Okada *et al.*, 1994). This technique measures the ability of cancer cells to invade through a cellular endothelial barrier and is analogous to commonly used assays that evaluate cancer cell invasion through extracellular matrix components (Albini *et al.*, 1987). Invasiveness is a hallmark of aggressive cancers, and this complex property has been the focus of intense investigation for some time. An emerging concept is that epithelial-to-mesenchymal transition (EMT)

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Abbreviations used: BLI, bioluminescent imaging; EMT, epithelial-to-mesenchymal transition; HMVEC-L, human microvascular endothelial cell from lung; qPCR, quantitative PCR.

may underlie the invasive characteristics of adenocarcinomas and has been proposed to regulate invasive behavior of cancer cells at the tumor–stroma interface (Berk *et al.*, 2007). However, whether EMT plays a role in transendothelial migration or extravasation of cancer cells has not been directly investigated. Here we sought to gain insight into the process of prostate cancer cell transendothelial migration by isolating variants of PC-3 cells with enhanced transendothelial migration in vitro and characterizing their migratory properties. We show that transendothelial cell migration selects for prostate cancer cells that have undergone a ZEB1-dependent EMT, whereas cell surface molecules that have previously been implicated in cancer cell transendothelial migration were primarily down-regulated or unchanged.

MATERIALS AND METHODS

Cell Lines

PC-3, LNCaP, 22Rv1, and DU145 cells were obtained directly from the ATCC (Manassas, VA). C42b cells were obtained from the laboratory of Dr. Michael Cohen (The University of Iowa, Iowa City, IA). Cell lines were stably transduced with a luciferase-expressing retroviral vector and were grown in the ATCC-recommended medium (GIBCO, Rockville, MD) supplemented with 10% FBS (Hyclone, Logan UT) and 1 mM nonessential amino acids (GIBCO) as described previously (Drake *et al.*, 2005). Primary human microvascular endothelial cells from lung (HMVEC-L; Lonza, Basel, Switzerland) were grown in EGM-2MV medium (Lonza) supplemented as indicated by the manufacturer. All cells were grown at 37°C and 5% CO₂. TEM4-18, TEM2-5 (PC-3 cell lines isolated after migration across an HMVEC-L monolayer), GS689.Li, and GS694.LAd cells were grown in DMEM/F12 medium supplemented with 400 µg/ml G-418. GS689.Li and GS694.LAd cell lines are twice passaged in vivo and derived from the PC-3 cell line JD549.Ki (a single passaged in vivo cell line that was isolated from a kidney tumor). GS689.Li and GS694.LAd cell lines are isolated from tumors that formed in the liver and adrenal gland, respectively, in *scid* mice. For ZEB1 knockdown, pLKO1 lentiviral vectors targeting human ZEB1 were purchased from Open Biosystems (Huntsville, AL; TRC collection). 293FT cells were transfected with this construct along with lentiviral second-generation plasmids psPAX2 and VSVg (kind gifts from the Trono lab) and culture supernatant from these cells was used to infect TEM4-18 cells for 8 h followed by puromycin selection (1 µg/ml). Stable TEM4-18 cells were then cloned using limiting dilution and assessed for ZEB1 knockdown before functional assays. We were able to achieve ~80% or greater knockdown of ZEB1 using two independent constructs with the following sequences: construct 63 (5'-ccgggCAACAATCAAGAGGTAAActcgagTTTAACTCTGTATTGTGctttt-3') and construct 65 (5'-ccgggCTCTCTGAAAGAACACATTActcgagTAATGTGTCTTTCAGAGAGgtttt-3').

Small Interfering RNA Transfection

For transient N-cadherin knockdown, TEM4-18 cells were plated at a density of $1-2 \times 10^5$ in a six-well plate 1 d before transfection. ON-TARGETplus SMARTpool N-cadherin or ON-TARGET plus nontargeting control small interfering RNAs (siRNAs; 20 µM, DharmaconResearch, Boulder, CO) were diluted in Opti-MEM reduced serum media (Invitrogen, Carlsbad, CA) and allowed to incubate with Lipofectamine2000 transfection reagent (1 mg/ml, Invitrogen) for 20 min. siRNAs were then added to the cells to establish a final concentration of 50 nM, combined with 2 µg/ml Lipofectamine2000. Cells were extracted at 55 h for RNA and protein analysis.

Cell Migration Assays

Primary HMVEC-Ls (3.5×10^4 cells) were plated onto either collagen IV-coated (Sigma, St. Louis, MO) 8-µm 24-well transwell inserts (Corning, Corning, NY) or 8-µm 24-well fluoroblock transwell inserts (Fisher Scientific, Pittsburgh, PA) and allowed to form a monolayer over 3–5 d. Integrity of the endothelial monolayer was evaluated by measuring electrical resistance as follows: Using an EVOM Voltmeter (World Precision Instruments, Sarasota, FL) and Endohm-6 transwell insert cup (World Precision Instruments) resistance across the endothelial monolayer was measured on each transwell insert, with a target resistance of $>10 \Omega/\text{cm}^2$ before use in transendothelial migration assays. Before plating onto HMVEC-Ls, prostate cancer cells were detached with 0.48 mM Versene (Invitrogen) for 10–15 min. For the fluorescence-based assay, prostate cancer cells were treated with 5 µM 5-chloromethylfluorescein diacetate (CMFDA) (Molecular Probes, Eugene, OR) for 30 min before detachment with Versene. Cells were then resuspended in complete DMEM/F12 media, spun at $200 \times g$ for 5 min, and resuspended in EGM media at a concentration of 5×10^5 cells/ml. Prostate cancer cells (1×10^5 , 200 µl) were added onto the HMVEC-L monolayer and allowed to incubate for 18 h before analysis of

migration. A standard curve was performed by serial dilution of prostate cancer cells (10,000–20 cells) in a 96-well dish followed by bioluminescence imaging (BLI) in a Xenogen IVIS100 imaging system (Caliper Life Sciences, Mountain View, CA).

To assay migration using BLI, transwell inserts were placed into a new 24-well dish containing trypsin (400 µl, 10 min at 37°C). After 10 min, trypsin was neutralized with 600 µl of serum-containing DMEM/F12 medium, and each insert was washed with medium. Sample, 100 µl, in duplicate, from each well was then added to a black 96-well dish (Corning, Corning NY) followed by addition of 100 µl of luciferin (0.3 mg/ml). BLI was determined after a 5-min luciferin incubation. Cell quantification was performed by converting the BLI signal from the sample into the standard curve to derive the number and percent of total cells migrated. To assay migration using fluorescence, each insert was washed in PBS and fixed in 4% paraformaldehyde for 15 min. After fixation, each insert was excised with a scalpel blade and mounted on a slide, bottom side up. Cells were visualized using a Leica DM2500 fluorescent microscope (Deerfield, IL) using a Cy2 filter. Labeled cells were counted and averaged (five fields, $10\times$). Experiments were performed in triplicate, and the data presented herein represent one of three individual experiments.

For Matrigel invasion experiments, 24-well Matrigel invasion chambers (BD Biosciences, San Jose, CA) were prepared and hydrated according to manufacturer's instructions. After chamber preparation, cancer cells were processed as described previously for the transendothelial migration assay and plated at a density of 1×10^5 cells/well for 24 h. In some experiments we omitted HMVEC-L cells from the upper chamber to assess random migration or plated them on the lower chamber to assess chemotactic response of PC-3 and TEM4-18 cells.

Metastatic Colonization

We performed all procedures involving animals according to The University of Iowa Animal Care and Use Committee policies. Using a 27-gauge needle, we injected 200 µl (1×10^6) of PC-3 cell suspension into the tail vein into 5–8-wk-old male *scid* mice (Taconic Farms, Germantown, NY). We performed BLI in a IVIS100 imaging system (Caliper Life Sciences) as described previously (Drake *et al.*, 2005). Whole body tumor growth rates were measured as follows: A rectangular region of interest was placed around the dorsal and ventral images of each mouse, and total photon flux was quantified using Living Image software v2.50 (Caliper Life Sciences) with the units of photons per second. The dorsal and ventral values were then summed and plotted weekly for each animal. Total photon flux was quantified using Living Image software with the units of photons per second. The values were plotted weekly for each animal. To adjust for the fourfold difference in BLI intensity between PC-3 and TEM4-18 cells, we multiplied the photon flux in the TEM4-18 group accordingly.

Microarray Analysis

Total RNA was extracted from low passage PC3, TEM4-18, and TEM2-5 cells with TRIzol reagent (Invitrogen) followed by RNeasy (Qiagen, Chatsworth, CA) cleanup. Two separate total RNA samples of each cell line were prepared, processed, and analyzed. Samples were then sent to the University of Iowa DNA Core Facility for processing. RNA quality was assessed using the Agilent Model 2100 Bioanalyzer (Agilent Technologies, Wilmington, DE). Five micrograms of total RNA was processed for use on the microarray by using the Affymetrix GeneChip one-cycle target labeling kit (Affymetrix, Santa Clara, CA) according to the manufacturer's recommended protocols. Briefly, total RNA was converted to double-stranded cDNA using Superscript II Reverse Transcriptase (Invitrogen) and an oligo-dT primer linked to a T7 RNA polymerase binding site sequence. The amplified, labeled cRNA was produced in an in vitro transcription reaction using T7 RNA polymerase and biotinylated nucleotides. After removal of free nucleotides, cRNA yield was measured by UV260 absorbance. Labeled cRNA, 15 µg, was fragmented and combined with hybridization control oligomer (b2) and control cRNAs (BioB, BioC, BioD, and CreX) in hybridization buffer and hybridized with the Affymetrix Human Genome U133 Plus 2.0 GeneChip. After a 16-h incubation at 45°C, the arrays were washed, stained with streptavidin-phycoerythrin (Molecular Probes), signal amplified with anti-streptavidin antibody (Vector Laboratories, Burlingame, CA) using the Fluidics Station 450 (Affymetrix). Arrays were scanned with the Affymetrix Model 3000 scanner, and data were collected using the GeneChip operating software (GCOS) v1.4. Data were analyzed using Partek Genomics Suite (Partek, St. Louis, MO). Signal intensities were normalized by Partek RMA. Statistical difference was calculated by two-way ANOVA analysis with false discovery rate (FDR). An FDR of $q < 0.05$ was used as a significance threshold; 752 probe sets (1.38%) showed ± 2 -fold change between PC-3 and the TEM4-18/TEM2-5 cell lines. For gene ontology analysis comparing PC-3 and TEM4-18, an FDR of $q < 0.1$ was used and genes showing ± 2 -fold change were submitted to GOMiner. Results were reported $p < 0.05$. The raw microarray data set was submitted online to the GEO repository (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE14405>) with accession number GSE14405.

Flow Cytometry Analysis

Cells were detached using 2 ml of 0.48 mM Versene/10-cm dish and incubated for 10–20 min. The cells were then harvested, resuspended in 10 ml of

serum-containing DMEM/F12 medium, and spun at $200 \times g$ for 5 min. Cells, 5×10^5 per tube, were placed into 1.5-ml Eppendorf tubes and spun down at $200 \times g$ at 4°C for 5 min. Supernatant was removed and $50 \mu\text{l}$ FACS buffer (PBS + 0.02% sodium azide + 5% BSA) + E-cadherin (1:100, R&D Systems, Minneapolis, MN) antibodies were added to the cells. The cells were then incubated for 20 min on ice in the dark, washed with 1 ml of fluorescent-activated cell sorting (FACS) buffer, and pelleted at $200 \times g$ for 5 min at 4°C . FACS buffer + secondary antibody (1:100, goat anti-mouse FITC, Chemicon, Temecula, CA) was added to the cells and incubated for 20 min on ice in the dark. Cells were washed again with 1 ml of FACS buffer followed by a 5-min spin at $200 \times g$ for 4°C , resuspended in $400 \mu\text{l}$ of FACS buffer, and transferred to a 12×75 -mm polystyrene FACS tube (BD Biosciences). Samples were analyzed using the Becton Dickinson FACScan at The University of Iowa Flow Cytometry Core Facility.

Quantitative PCR

Human ZEB1 forward primer: 5'-GCACCTGAAGAGGACCAGAG-3', reverse primer: 5'-TGCATCTGGTGTCCATTT-3'; human E-cadherin forward primer: 5'-GCTGAGCTGGACAGGGAGGA-3', reverse primer: 5'-ATGGGGCGTGTGTCATTAC-3'; human GAPDH forward primer: 5'-CCATGTCGTCATGGGTGTG-3', reverse primer: 5'-CAGGGGTGCTAAGCAGT-TGG-3'. Quantitative PCR (qPCR) analysis was performed as described previously (Svensson *et al.*, 2007). Relative expression values were calculated using the comparative Ct method (Pfaffl, 2001).

Western Blot Analysis

Mouse anti-human monoclonal E-cadherin antibody was purchased from R&D Systems and rabbit anti-rat ZEB1 polyclonal antibody was a kind gift from Dr. Douglas Darling (University of Louisville; Costantino *et al.*, 2002). We prepared 2% SDS protein lysates followed by separation by SDS-PAGE and transfer to an Immobilon-FL polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). The membrane was blocked in Odyssey Blocking Buffer (Li-Cor Biosciences, Lincoln, NE), diluted 1:1 in PBS, for 1 h at room temperature followed by incubation of either E-cadherin (1:2000) or ZEB1 (1:3000) primary antibodies overnight at 4°C in Odyssey Blocking Buffer with 0.1% Tween-20. The membrane was washed three times for 5 min in PBS followed by incubation with either Odyssey goat anti-mouse IRDye 680 (1:10,000, Li-Cor Biosciences) or Odyssey goat anti-mouse IRDye 800CW (1:10,000, Li-Cor Biosciences) secondary antibodies for 1 h at room temperature in Odyssey Blocking Buffer with 0.1% Tween-20. The membrane was then rinsed three times for 5 min in PBS followed by exposure using the Odyssey Infrared Imaging System (Li-Cor Biosciences).

Immunofluorescence

Cells ($n = 50,000$) were plated onto poly-L-lysine-coated glass coverslips (Sigma) in 24-well dishes (Nunc, Naperville, IL) and allowed to grow to near confluence. For ZEB1 staining, cells were then washed two times in PBS followed by fixation with fresh 4% paraformaldehyde for 15 min at room temperature (Darling *et al.*, 2003). Before blocking, cells were permeabilized

Figure 1. Selection and characterization of TEM4-18 cells. (A) Schematic representing the derivation of TEM4-18 cells from PC-3 cells after two rounds of passage through HMVEC-L monolayers. (B) Phase contrast image ($200\times$) displaying the morphology of PC-3 compared with TEM4-18 cells plated at clonal density. Insets show colony morphology. TEM4-18 cells exhibit enhanced transendothelial migration compared with PC-3 cells as assessed by BLI (C) or direct cell counts (D). In contrast, TEM 4-18 cells exhibit less migration across a collagen IV-coated insert without endothelial cells (E) through Matrigel (F), or in a chemotaxis assay where HMVEC-L cells were plated in the lower chamber (G). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus PC-3 cells.

with 0.1% Triton X-100 for 30 min. Cells were then washed two times in PBS followed by addition of 300 μ l of blocking solution (1% BSA, 0.1% sodium azide in PBS) to each well for 1 h. After blocking, primary ZEB1 antibody was diluted 1:500 in blocking solution, and 300 μ l was added to the cells for 1 h at room temperature on a rocker. Cells were washed three times in PBS followed by addition of 300 μ l of goat anti-rabbit (1:1000, Jackson ImmunoResearch Laboratories, West Grove, PA) secondary antibody and DAPI (1:5000, Sigma) in blocking solution for 30 min at room temperature on a rocker. Cells were washed three times in PBS and cover-slipped, and images were taken with a Leica DM2500 fluorescent microscope (Deerfield, IL).

RESULTS

Selection of Cells Exhibiting Enhanced TEM

To understand the process of transendothelial migration of prostate cancer cells, we adapted an assay commonly used to study leukocyte diapedesis (Moreland and Bailey, 2006). We plated HMVEC-L cells on a porous membrane and allowed these cells to reach confluence and establish cell-cell junctions. We monitored the integrity of this monolayer by dye exclusion, electrical resistance, immunohistochemistry, and immunofluorescence and determined the optimal conditions for measuring the migration of prostate cancer cells across this barrier (Supplemental Figure S1). We reasoned that serial passage of PC-3 cells across this barrier might select for cells with enhanced transendothelial migration characteristics and designated this variant PC-3 cell line TEM4-18 (Figure 1A). Initially, we noticed differences in TEM4-18 cell morphology. Although the PC-3 cells formed more organized epithelial colonies, TEM4-18 cells were elongated and spindle-shaped (Figure 1B). TEM4-18 cells exhibit about fivefold greater transendothelial migration than PC-3 cells as measured using BLI (Figure 1C) and fluorescence-based direct cell counts (Figure 1D). An independent isolation of PC-3 variants by this method yielded similar results (Supplemental Figure S3).

To determine whether TEM4-18 cells exhibit generally enhanced migratory or invasive properties, we performed additional *in vitro* assays. Although TEM4-18 cells were better able to cross an endothelial cell barrier, surprisingly, they exhibited far less migration across transwell membranes without endothelial cells (Figure 1E) and less invasion through Matrigel (Figure 1F). To determine whether secreted factors from endothelial cells elicit the enhanced migration of TEM4-18 cells we plated HMVEC-L cells in the lower chamber. Although the presence of endothelial cells increased migration of both PC-3 and TEM4-18 cells, the latter still exhibited less migration than PC-3 cells (Figure 1G). We next compared the migration/invasion properties of TEM4-18 cells with PC-3-derived cell lines selected for metastatic tumor growth *in vivo* (Figure 2, A–C). These cell lines (GS689.Li and GS694.LAd) were isolated from the liver and adrenal gland after intravenous injection of JD549.Ki cells (see *Materials and Methods*). TEM4-18 cells, selected *in vitro*, and GS689.Li and GS694.LAd cells, selected *in vivo*, showed the same profile of behavior: enhanced transendothelial migration (Figure 2A), reduced migration across transwell filters alone (Figure 2B), and poor invasion through Matrigel (Figure 2C) relative to the parental PC-3 cell line. Taken together these results indicate that TEM4-18 cells exhibit enhanced migration only when in direct contact with endothelial cells and are not generally more migratory or invasive. Moreover, TEM4-18 cells share the same profile of migratory/invasive behaviors with cell lines isolated from metastatic tumors, suggesting that cells selected for transendothelial migration *in vitro* might have more aggressive behavior *in vivo*.

Figure 2. *In vivo*-passaged PC-3 cells display characteristics similar to TEM4-18 cells in *in vitro* cell migration assays. Transendothelial migration is enhanced in TEM4-18 and in twice-passaged *in vivo* PC-3 cell lines (GS689.Li and GS694.LAd) relative to the parental PC-3 cells (A), but migration across a collagen IV-coated insert without HMVEC-L cells (B) or through Matrigel (C) is reduced. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus PC-3 cells.

TEM4-18 Cells Exhibit Enhanced Metastatic Colonization

We next examined the ability of TEM4-18 cells to form tumors *in scid* mice after intravenous injection. We injected PC-3, TEM4-18, or JD549.Ki cells via tail vein and monitored bioluminescence signal for tumor formation and growth. JD549.Ki metastatic colonization was greatly enhanced compared with PC-3 cells (Figure 3, F vs. D, respectively) in accord with previous findings (Pettaway *et al.*, 1996). TEM4-18 cells demonstrated an intermediate phenotype between JD549.Ki and PC-3 cells (Figure 3E), as evident in measurements of both whole body tumor growth (Figure 3, G–J) and tumor incidence (Figure 3K). There was no correlation between the intrinsic growth rates of the cells or growth rates of subcutaneous xenografts of PC-3 and TEM4-18 cells and their behavior in the metastatic colonization model, and these were not significantly different from one another (Supplemental Figure S2). This argues that enhanced growth rates, *per se*, do not account for the differences in the behavior of these cell lines in the metastatic colonization model. Thus, although TEM4-18 cells shared *in vitro* migration properties with *in vivo*-passaged PC-3 cell lines, the latter are more aggressive than TEM4-18 cells, which in turn are more aggressive than parental PC-3 cells in a mouse model of metastatic colonization. These results are consistent with the interpretation that TEM4-18 cells selected for transendothelial migration *in vitro* might exhibit enhanced extravasation *in vivo*, thereby increasing metastatic colonization frequency; but additional selective pressures must be exerted *in vivo*, such as the ability to survive

Figure 3. TEM4-18 cells exhibit enhanced tumor formation in vivo. (A–F) BLI was used to quantify tumor burden in *scid* mice ($n = 10$ /cell line) injected, via tail vein, with either PC-3 (A and D), TEM4-18 (B and E), or JD549.Ki (C and F), an in vivo–derived PC-3 cell line derived from a kidney metastasis, cells. TEM4-18 cells displayed an intermediate phenotype in which they formed tumors better than PC-3 cells, but were not as efficient as JD549.Ki cells. Each panel represents the images of five mice taken at day 0 (A–C) and either at day 112 (D and E) or day 63 (F) after injection. It is important to note that the differences in luminescence output between PC-3 and TEM4-18 cells at day 0 (A and B) reflects a fourfold less bioluminescence signal in TEM4-18 cells relative to PC-3. (G–I) Tumor growth rates were quantified as described in *Materials and Methods* and graphed individually for PC-3 (G), TEM4-18 (H), and JD549.Ki (I). (J) Average tumor growth rates were quantified and graphed at the times indicated after injection. JD549.Ki cells formed tumors at a significantly greater rate and frequency than either TEM4-18 or PC-3 cell lines (J), $p < 0.05$ by day 42). Accounting for the fourfold reduced bioluminescence intensity observed in TEM4-18 cells, they also exhibit enhanced tumor growth compared with PC-3, but less than that observed in JD549.Ki (J), $p < 0.05$). (K) Kaplan-Meier analysis of tumor formation also shows that mice injected with JD549.Ki and TEM4-18 cells exhibit greater tumor formation than PC-3 cells. * $p < 0.05$ versus PC-3 tumors.

Figure 4. Selection of E-cadherin-negative cells after transendothelial migration. (A) qPCR analysis reveals a dramatic down-regulation of E-cadherin transcript in TEM4-18, GS689.Li, and GS694.LAd cells. Western blot (B) and flow cytometry analysis (C and D) confirms that total and cell surface E-cadherin protein, respectively, is also greatly reduced in TEM4-18, GS689.Li, and GS694.LAd relative to PC-3 cells. This analysis shows that E-cadherin-negative cells exist as a subpopulation in PC-3 cells. Prospective isolation of E-cadherin-negative (PC-3neg) and E-cadherin-positive (PC-3E) cells by fluorescence activated cell sorting (E) demonstrates the PC-3neg cells exhibit enhanced transendothelial migration compared with PC-3E cells. * $p < 0.05$ versus PC3E cells and ** $p < 0.01$ versus PC-3 cells.

and grow at metastatic organ sites, resulting in the still more aggressive in vivo-passaged cell lines.

Transendothelial Migration Selects for an E-Cadherin-Negative Subpopulation of PC-3 Cells

The morphological differences of TEM4-18 cells compared with PC-3 cells (Figure 1B) suggested that TEM4-18 cells may have undergone EMT. A hallmark of such is the loss of E-cadherin expression. Indeed, TEM4-18, as well as in vivo-passaged GS689.Li and GS694.LAd cells, lack appreciable E-cadherin expression at the mRNA (Figure 4A) and protein levels, both in whole cell lysates and on the cell surface (Figure 4, B–D). Flow cytometry of PC-3 cells show that there is a distinct subpopulation (~25%) of E-cadherin-negative cells, consistent with a prior report (Rokhlin and Cohen, 1995). We confirmed this finding in unmodified PC-3 cells within a few passages from the initial ATCC stock, establishing that the addition of a luciferase retroviral expression construct did not alter the ratio of E-cadherin-positive and -negative cells (data not shown). This suggested that the about fivefold increase in migration observed in TEM4-18 cells (Figure 1, C and D) could be accounted for by the selective enrichment of this E-cadherin-negative subpopulation. To test this, we prospectively isolated E-cadherin-positive (PC-3E) and -negative (PC-3neg) cells by FACS and evaluated these cells in the transendothelial migration assay (Figure 4E). PC-3neg cells migrated about sixfold better than PC-3E cells (Figure 4F), consistent with the suggestion that enhanced transendothelial migration is a property of the preexisting E-cadherin-negative subpopulation.

Microarray Analysis Reveals a Pattern of Gene Expression in TEM4-18 Cells Consistent with an EMT

Using microarray analysis, we sought to define the differences in gene expression between TEM4-18 and PC-3 cells. We compared PC-3 cells to TEM4-18 and an independent isolate from the transendothelial migration assay: TEM2-5 cells (see Supplemental Figure S3). A total of 487 genes were significantly regulated (0.05 FDR): greater than twofold (249 up-regulated; 238 down-regulated). Interestingly, gene ontology analysis with GOMiner (Zeeberg *et al.*, 2003), comparing differentially expressed genes between PC-3 and TEM4-18, showed that “leukocyte_chemotaxis” ($p = 0.015$) and “neutrophil_chemotaxis” ($p = 0.030$) ontologies scored significant (out of 14 total; $p < 0.05$) including the down-regulated genes *IL1B*, *SYK*, *TGFB2*, *IL8*, and *CXCL16*. This suggested that, contrary to expectations, the expression of genes that are involved in leukocyte trafficking and have been implicated in cancer cell extravasation might be selected against in the TEM cell populations. To investigate this possibility in more detail, we further evaluated the microarray and performed FACS analysis for genes that have been previously implicated in cancer cell extravasation. In a set of genes involved in cytokine/chemokine signaling and cell adhesion, the microarray showed that the expression of these genes was either unchanged or down-regulated (Supplemental Figure S4). We confirmed and extended these findings by FACS analysis. This showed that, with the notable exception of integrin $\beta 3$, which was up-regulated in TEM4-18 cells and has been previously implicated in transendothelial migration of PC-3 cells (Romanov and Goligorsky, 1999; Wang *et al.*, 2005), there was either little change in

Figure 5. Microarray analysis reveals an up-regulation of ZEB1 and high ZEB1 correlates with enhanced transendothelial migration in a panel of prostate cancer cell lines. (A) Microarray analysis of TEM4-18 and TEM2-5 (a biological replicate of TEM4-18, see Supplemental Figure S3) reveals an up-regulation of EMT regulators, ZEB1, and Twist2, whereas other known EMT regulators are unchanged, relative to PC-3 cells. Scatter plot analysis reveals a distinct correlation between up-regulation of ZEB1 and down-regulation of epithelial markers (B). Yellow squares (EMT regulators), orange triangles (mesenchymal markers), and maroon circles (epithelial markers). (C) Immunofluorescence analysis of ZEB1 in PC-3 and TEM4-18 reveals nuclear localization in about 25% of PC-3 cells and 100% of TEM4-18 cells. (D) A panel of prostate cancer cell lines were plated onto HMVEC-Ls and assayed for their transendothelial migration. DU145 cells showed the greatest transendothelial migration, which corresponded to the highest level of ZEB1 expression (D, bottom chart). Fold expression data are based on qPCR analysis. * $p < 0.05$ versus C42b and *** $p < 0.001$ versus DU145 cells. Scale bar, 25 μm .

expression, or in the case of integrin $\beta 4$ and MUC1, markedly reduced expression on the cell surface (Supplemental Figures S4 and S5, F, G, and J). This analysis included the E-selectin ligands sialyl-Lewis^x (SLe^x) and sialyl-Lewis^a (SLe^a) antigens, which were modestly down- and up-regulated, respectively, in TEM4-18 cells (Supplemental Figure S5, D and H). In sum, although many cell surface adhesion molecules that have previously been implicated in transendothelial cell migration were indeed expressed in TEM4-18 cells (including integrins $\alpha 4$, $\alpha 6$, αV , and $\beta 1$; CD44; and SLe^a, SLe^x antigens), differential expression of these molecules alone is unlikely to account for the enhanced transendothelial migration ability of TEM4-18 cells.

Because our data suggests that TEM4-18 cells underwent EMT we focused our attention on those genes implicated in regulating EMT. Figure 5A shows the relative expression levels of a set of transcription factors thought to regulate EMT. Only two transcription factors, ZEB1 (a.k.a. δ -EF1,

TCF8, and AREB6) and Twist2, showed differential regulation between PC-3 and TEM4-18 cells (Figure 5A). qPCR analysis confirmed up-regulation of both ZEB1 and Twist2 mRNA in TEM4-18 cells, as well as in vivo-passaged lines GS689.Li and GS694.LAd (Supplemental Figure S4, B and C, respectively), suggesting that either of these transcription factors may be responsible for the EMT evident in TEM4-18 cells. To clarify the roles of these transcription factors, we examined the expression of additional genes in the microarray data set. A previous study in which ZEB1 was knocked down in breast cancer cells identified a set of up-regulated epithelial genes that are putative targets of ZEB1 repression (Aigner *et al.*, 2007). We found that 27 of 37 of these genes were significantly down-regulated in TEM4-18 cells consistent with the hypothesis that ZEB1 expression actively represses the expression of these genes either directly or indirectly (Figure 5B, Supplemental Figure S6, A, D, and E). For ZEB1 to function as a transcriptional repressor, it must be

localized to the nucleus. Therefore, we evaluated immunofluorescence staining of ZEB1 in both PC-3 and TEM4-18 cells. Nuclear ZEB1 staining was apparent in all TEM4-18 cells, whereas only some PC-3 cells showed ZEB1 nuclear staining, indicating this protein is properly localized and potentially functional (Figure 5C). As would be expected from the E-cadherin flow cytometry results (Figure 4C), the PC-3 cell population showed ~25% ZEB1-positive staining in the nucleus (28/114 cells, 24.6%), again indicating that the TEM4-18 cells were derived from the E-cadherin-negative population.

On the basis of our findings that ZEB1 expression is associated with transendothelial migration and EMT in PC-3 cells, we investigated whether high ZEB1 expression is correlated with transendothelial migration in other prostate cancer cell lines. We compared androgen-dependent LNCaP cells, which do not exhibit metastatic colonization in mice, with C4-2b, a LNCaP derivative selected for growth in bone, and androgen-responsive 22Rv1 and androgen-independent DU145 cells, both of which exhibit aggressive metastatic colonization. High *ZEB1* expression correlated with high transendothelial migration in DU145 cells, whereas LNCaP, C42b, and 22Rv1 cells express relatively lower levels of *ZEB1* and show much reduced transendothelial migration (Figure 5D). Repeated passage of 22Rv1 cells did not result in elevated transendothelial migration (data not shown). Further, *ZEB1* expression inversely correlated with *E-cadherin* expression in DU145 cells (Figure 5D). This data suggests that *ZEB1* expression is highly correlated with transendothelial migration in at least one other prostate cancer cell line, but that other prostate cancer cell lines that exhibit aggressive metastatic colonization, particularly 22Rv1, may not depend on *ZEB1* for their aggressive behavior.

Transendothelial Migration of PC-3 Cells Depends on *ZEB1* and *N-Cadherin*

Our initial attention focused on *Twist2* and *ZEB1* as potential mediators of EMT in PC-3 cells, but siRNA-mediated knockdown of *Twist2* did not affect *E-cadherin* expression or morphology of TEM4-18 cells (data not shown). To test the role of *ZEB1* in transendothelial migration, we stably integrated a lentiviral shRNA targeting the *ZEB1* mRNA into TEM4-18 cells. We identified two independent shRNAs capable of reducing *ZEB1* protein levels to ~20% or less than those cells expressing a control shRNA. For each shRNA construct, we characterized two independent clones (Figure 6A, top panel). Consistent with our expectations, *E-cadherin* was induced in *ZEB1*-knockdown clones (Figure 6A, middle panel), interestingly, though, not to the levels observed in PC-3E cells. Further, cell morphology in *ZEB1*-knockdown cells (Figure 6, B and C) reverted from an elongated, spindle-shaped morphology apparent in the TEM4-18 control clones (Figure 6B) to a more typical epithelial morphology (Figure 6C). Finally, we found that *ZEB1*-knockdown in TEM4-18 cells displayed a significant reduction in transendothelial migration when compared with the control cells (Figure 6D). This data indicates that *ZEB1*, a regulator of EMT, plays an important role in transendothelial migration of prostate cancer cells.

N-cadherin is often a marker of EMT and has also been implicated in transendothelial migration. Therefore we investigated its expression in TEM4-18 cells. Notably, as with other cell adhesion molecules implicated in transendothelial migration *N-cadherin* was unchanged in TEM4-18 cells, when compared with PC-3 cells (Supplemental Figure S5K). We determined whether HMVEC-L cells express *N-cadherin*

Figure 6. Knockdown of *ZEB1* partially restores the epithelial phenotype and reduces transendothelial migration. (A) Western blots of both *ZEB1* and *E-cadherin* reveal that stable knockdown of *ZEB1* correlates to reexpression of *E-cadherin* in two separate clones from two independent shRNA constructs. Morphological changes are observed in TEM4-18 cells after *ZEB1* knockdown (B and C). The cells change from an elongated mesenchymal (shControl#3, B) to a more rounded epithelial-like phenotype (sh63#4, C). The yellow box in B and C represents cell tracings from selected cells. (D) Reduced transendothelial migration is observed after *ZEB1* knockdown. PC3E and TEM4-18 cells are shown for reference, and we compared the *ZEB1* knockdown to control clones. * $p < 0.05$, *** $p < 0.001$ versus shControl#3 or shControl#5 cells.

and, as expected, *N-cadherin* is primarily localized diffusely on the surface of HMVEC-L cells, with some staining in VE-cadherin-positive endothelial cell-cell junctions (Figure 7, A–C). To test if *N-cadherin* in TEM4-18 cells is required for transendothelial migration, we silenced this gene using siRNAs. We were able to knockdown cell surface *N-cadherin* to ~85% of the controls, as assessed by flow cytometry (Figure 7D). We found that TEM4-18 cells with *N-cadherin* knockdown displayed about a threefold reduction in transendothelial migration compared with the nontargeting siRNAs (Figure 7E, $p < 0.05$).

DISCUSSION

This work presents two primary findings: 1) transendothelial migration selects for PC-3 cells that exhibit EMT, and 2) *ZEB1* regulates EMT and is required for transendothelial migration of a subpopulation of PC-3 cells. Migration and invasion of these cells in other contexts were greatly reduced compared with parental PC-3 cells, indicating that direct contact with endothelial cells (or their extracellular matrix) is required to reveal their enhanced invasive properties. EMT was originally described as a transient process neces-

Figure 7. Knockdown of N-cadherin reduces transendothelial migration of TEM4-18 cells. Immunofluorescence shows VE-cadherin staining at the HMVEC-L cell junctions (A), whereas N-cadherin expression is localized diffusely on the plasma membrane, with minimal staining at the cell junctions (B). Flow cytometry analysis also confirms cell surface N-cadherin expression in HMVEC-L cells (C). Knockdown of N-cadherin in TEM4-18 cells using siRNAs reduced N-cadherin expression by 85%, as indicated by flow cytometry (D). CMFDA-labeled TEM4-18 cells transfected with either nontargeting control (siCtrl) or N-cadherin (siNCad) siRNAs were plated onto HMVEC-L cells, and transendothelial migration was assessed 18 h later. Transendothelial migration was reduced by ~70% after N-cadherin knockdown when compared with the controls (E). * $p < 0.05$ versus TEM4-18 siControl (siCtrl) cells. Scale bar, 10 μm .

sary for the migration of epithelial cells during development and, although not without controversy, EMT has been implicated in invasive behaviors of adenocarcinomas (Berk *et al.*, 2007). EMT has been associated with locally invasive behaviors at the tumor–stroma interface, but not specifically with transendothelial migration or extravasation. Our data now indicate that EMT facilitates yet another step in the metastatic cascade.

EMT is associated with loss of epithelial and gain of mesenchymal characteristics, resulting in increased invasive potential. EMT in cancer cells is regulated by a number of transcription factors including more notably Snail1 and Twist1 (Battle *et al.*, 2000; Cano *et al.*, 2000; Yang *et al.*, 2004). Our studies indicate that another EMT regulator ZEB1 is responsible for repression of the epithelial genes, including E-cadherin, in prostate cancer cells. Prior studies in breast and colon cancer cells identified a group of epithelial genes under direct or indirect control of ZEB1 (Aigner *et al.*, 2007; Spaderna *et al.*, 2008). We show that many of these epithelial genes are down-regulated in high ZEB1-expressing TEM4-18 prostate cancer cells, and conversely their expression is increased in ZEB1-silenced cells. Micro-RNAs of the miR-200 family repress ZEB1 and increase E-cadherin expression (Hurteau *et al.*, 2007; Burk *et al.*, 2008; Gregory *et al.*, 2008; Korpil *et al.*, 2008; Park *et al.*, 2008). Thus reduced miR-200 family expression in TEM 4-18 cells might result in increased levels of ZEB1, perhaps engaging a negative feedback loop (Bracken *et al.*, 2008). Other possible mechanisms involved in inducing ZEB1 expression in prostate cancer cells include insulin-like growth factor I (Graham *et al.*, 2008). ZEB1 knockdown only partially restores E-cadherin expression in TEM4-18 cells. This could be due to incomplete silencing of ZEB1, or alternatively, other EMT transcriptional regulators, such as SIP1 or Twist1, may compen-

sate for the loss of ZEB1 maintaining lower levels of E-cadherin. The expression of these factors could be coordinately regulated by a loss of E-cadherin expression, pointing to complex relationships between the transcriptional regulators of EMT and their targets (Onder *et al.*, 2008). Although a significant number of epithelial genes were down-regulated in TEM4-18 cells, many mesenchymal genes remained unchanged. We measured both *N-cadherin* and *vimentin* mRNA levels using qPCR (data not shown) and only found *vimentin* to be slightly increased, whereas *N-cadherin* was unchanged when compared with the PC-3 parental population. Moreover, E-cadherin-positive PC-3 cells express N-cadherin as well. This suggests that PC-3 cells have already undergone a partial EMT, allowing the expression of a subset of mesenchymal genes, but that additional up-regulation of ZEB1 is required to fully repress the epithelial phenotype in these cells. Thus, in PC-3 cell cultures there are distinct subpopulations representing different points along a spectrum of changes associated with EMT. Discerning how the various regulators of EMT contribute to this spectrum is an important area of future research.

We were surprised to find that a number of cell adhesion molecules previously implicated in transendothelial migration of cancer cells were not enriched in TEM4-18 cells. Among these only $\beta 3$ integrin, which has already been implicated in transendothelial migration of PC-3 cells (Wang *et al.*, 2005), was increased whereas expression of others was either unchanged or down-regulated such as $\beta 4$ integrin, perhaps reflecting the loss of epithelial identity, and MUC1. This may be due to the fact that TEM4-18 cells were selected in a static assay (no shear flow) so that tight adhesion between the cancer cells and endothelium was not necessary. Alternatively, this may reflect the different modes of cancer cell migration. It was striking that the global gene

expression pattern showed reduced expression of genes associated with leukocyte chemotaxis, suggesting that EMT in these cells may promote mesenchymal versus amoeboid, leukocyte-like migration (Friedl and Wolf, 2003). N-cadherin is considered a marker of EMT and has also been implicated in transendothelial migration of melanoma cells where it is involved in homophilic binding to N-cadherin expressed on endothelial cells and may promote cell adhesion, cell signaling, or both (Qi *et al.*, 2005, 2006). We show here that N-cadherin is necessary for transendothelial migration of TEM4-18 prostate cancer cells. However, the expression of N-cadherin is not sufficient for promoting transendothelial cell migration as it is equivalently expressed in the parental PC-3 cells, which are poor at transendothelial cell migration. Thus, it is likely that ZEB1 induces further changes in PC-3 cells, which allows for efficient transendothelial migration in an N-cadherin-dependent manner.

Our results clearly demonstrate that ZEB1 is required for efficient transendothelial migration of TEM4-18 cells although the mechanism(s) by which ZEB1 contributes to transendothelial migration are not yet clear. Perhaps repression of epithelial characteristics per se is permissive for transendothelial migration, e.g., loss of E-cadherin expression may enable N-cadherin-dependent functions. Alternatively, ZEB1 may either directly or indirectly control the expression of genes(s) that are required for transendothelial migration. N-cadherin is apparently not a candidate because it is expressed in PC-3E cells, which lack ZEB1 expression, and neither is $\beta 3$ integrin because its expression was unchanged in ZEB1-silenced cells (data not shown). This does not preclude the possibility that ZEB1 influences the function of those molecules or their connection to cell motility. Interestingly, a survey of differentially expressed genes in cell lines competent for transendothelial migration from a large panel of cell lines from different tumor types showed both up-regulation of $\beta 3$ integrin and down-regulation of many of the same epithelial genes that are likely regulated by ZEB1, suggesting that ZEB1 may influence transendothelial migration in other cancers (Bauer *et al.*, 2007). ZEB1 is also highly expressed in at least one other prostate cancer cell line, DU145, which exhibits high levels of transendothelial migration and aggressive metastatic colonization in vivo. However, in another prostate cancer cell line, 22Rv1, ZEB1 expression was much lower, and these cells did not exhibit robust transendothelial migration. Repeated passage of 22Rv1 cells did not result in elevated transendothelial migration in this cell line, yet we have previously shown that this cell line exhibits robust metastatic colonization (Drake *et al.*, 2005). This indicates that ZEB1 expression and increased transendothelial migration are not obligate features of prostate cancer cells that exhibit robust metastatic colonization in vivo and points to alternative modes that mediate metastatic colonization. Nevertheless, loss of E-cadherin expression and its association with poor prognosis is well documented in prostate cancer, consistent with the possibility that ZEB1 may play a role in this process (Tomita *et al.*, 2000). Recent studies have shown that ZEB1 expression is correlated with high ($n = \geq 8$) Gleason grade prostate adenocarcinomas, indicating that ZEB1 is a marker of an aggressive prostate cancer phenotype in patients (Graham *et al.*, 2008). Further investigation is warranted to define the role of ZEB1 in prostate cancer progression.

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